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Influence of Cell Membrane Potential, and Selectivity of the Na^+/H^+ Exchanger and Cl^-/HCO_3^- Exchanger on the Intracellular Accumulation of Adriamycin

JUN-ICHI ASAUMI[1], SHOJI KAWASAKI[2], XIAN-SHU GAO[1], MASAHIRO KURODA[1] and YOSHIO HIRAKI[1]

[1]*Department of Radiology, Okayama University Medical School, and*
[2]*Department of Radiation Technology, School of Health Sciences, 2-5-1, Shikata-cho, Okayama 700, Japan*

Abstract. *Intracellular accumulation of adriamycin (ADR) has been reported to be influenced by cell membrane potential. We first evaluated intracellular accumulation of ADR and 3,3'-(di-n-hexyl)-2,2'-oxacarbocyanine iodide (NK-2280), an indicator of cell membrane potential, and found a good correlation between ADR and NK-2280 intracellular accumulation in several cell lines. This suggests that ADR accumulation may be influenced by cell membrane potential or the mechanisms of NK-2280 accumulation may be similar to those of ADR accumulation.*

Next, we observed the influence of the Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger on the intracellular accumulation of ADR and NK-2280, and found that ADR accumulation decreased with increasing concentratios of 3,5-diamino-6-chloro-N-(diaminomethylene)pyrazinecarboxamide (amiloride), an inhibitor of the Na^+/H^+ exchanger, and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), an inhibitor of the Cl^-/HCO_3^- exchanger, however, NK-2280 accumulation was increased by amiloride, and decreased by DIDS. The increased accumulation of NK-2280 induced by amiloride may be due to the increased cell membrane potential caused by the inhibition of H^+ ion efflux and Na^+ ion influx due to the inhibition of the Na^+/H^+ exchanger. The decreased accumulation of NK-2280 may be also due to the decreased cell membrane potential caused by the inhibition of Cl^- ion efflux due to the inhibition of the Cl^-/HCO_3^- exchanger by DIDS. However, the decreased rate caused by DIDS was greater than the increased rate caused by amiloride. Therefore, it is suggested that the decreased accumulation of NK-2280 by DIDS may be influenced by other factors apart from cell membrane potential. These results suggest that the Cl^-/HCO_3^- exchanger may be related to both ADR accumulation, and NK-2280 accumulation, and that the Na^+/H^+ exchanger may be related to ADR accumulation, but not NK-2280. This suggests that the Cl^-/HCO_3^- exchanger is of low selectivity.

Correspondence to: Dr. J. Asaumi, 37-117, Tatsumi, Okayama 700, Japan.

Key Words: Adriamycin, cell membrane potential, flow cytometry, Na^+/H^+ exchanger, Cl^-/HCO_3^- exchanger.

Adriamycin (ADR) is an antibiotics frequently used in the clinical treatment of cancer. Its cytotoxic action is influenced strongly by its intracellular accumulation (1,2). In the 1 μg-ADR-resistant strain of Ehrlich ascites tumor cells (EAT cells) grown in our laboratory, ADR accumulation decreased markedly due to enhancement of efflux of intracellular ADR by P-glycoprotein (3). ADR accumulation in the ADR-resistant strain was about 40% of that found in the parental cells. When ADR efflux was inhibited completely ADR accumulation in the resistant strain was of, about 80% of that found in the parental cells. Therefore, the decrease of about 20% in the ADR-resistant strain compared with that of the parental cells may be due to the inhibition of ADR influx (submitted for publication). The influx of ADR has been attributed to passive transport (4-6), but the cell membrane potential of anti-cancer drug resistant cells tends to be reduced. This reduction has been suggested as a useful indicator of resistance to anti-cancer drugs (7,8).

3,3'-(Di-n-hexyl)-2,2'-oxacarbocyanine iodide (NK-2280), a fluorescent substance, adsorbs to the cell membrane or is incorporate in it, according to cell membrane potential (9, 10). In the present study, we first observed the intracellular accumulation of ADR and NK-2280 in several cell lines, and found a good correlation between them.

On the other hand, we have reported that the ADR influx may be closely be related Na^+/H^+ exchanger and the Cl^-/HCO_3^- exchanger (4). 3,5-Diamino-6-chloro-N-(diaminomethylene)pyrazinecarboxyamide (amiloride) is known to be an inhibitor of the Na^+/H^+ exchanger, 4,4'-diisothio-

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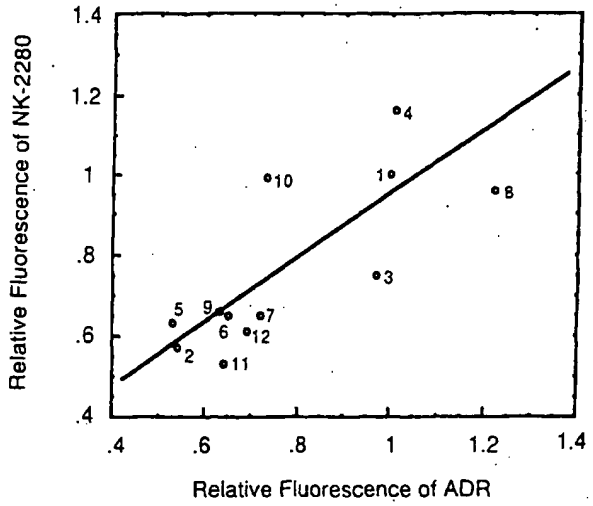

Figure 1. *Relationship between the intracellular accumulation of ADR and NK-2280 in the NIH3T3 cell line transfected with oncogenes by pSV2neo vector (2) The cells were treated with 10 μg/ml of ADR for 60 minutes, or 0.5 μM of NK-2280 for 20 minutes at 37° C, then the intracellular accumulation of ADR or NK-2280 was observed. Cell lines: 1=NIH3T3 cells, 2=NIH3T3 cells transfected with pSV2neo vector, 3-12 are NIH3T3 cell lines transfected with the following oncogenes by pSV2neo vector: 3=v-myc, 4=N-ras, 5=c-Ki-ras, 6=v-Ha-ras, 7=v-raf, 8=fgr, 9=v-src, 10=v-sis, 11=v-int-2, 12=v-erbB (Reference no. 2)*

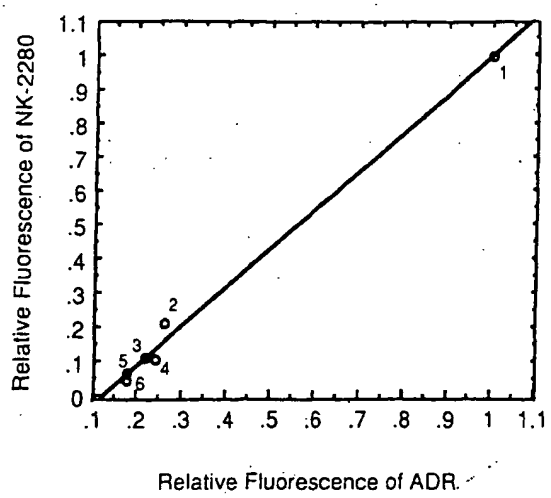

Figure 2. *Relationship between the intracellular accumulation of ADR and NK-2280 in the wild EAT cells and their 0.2, 0.5, 1, 5, 10 μg ADR-resistant strains.*
The methods was carried out as shown Figure 1, then the intracellular accumulation of ADR or NK-2280 was examined.
Cell lines: 1=wild type Ehrlich ascites tumor cells, 2=0.2 μg ADR-resistant strain, 3=0.5 μg ADR-resistant strain, 4=1 μg ADR-resistant strain, 5=5 μg ADR-resistant strain, 6=10 μg ADR-resistant strain.

cyanatostilbene-2,2'-disulfonic acid (DIDS) is an inhibitor of the Cl^-/HCO_3^- exchanger (11-13). Next, we observed the influence of amiloride, or DIDS on the intracellular accumulation of ADR and NK-2280 in Ehrlich ascites tumor cells (EAT cells), and the 1 μg ADR-resistant strain. We found that the Cl^-/HCO_3^- exchanger was related to both ADR and NK-2280 on the intracellular accumulation, and the Na^+/H^+ exchanger was related to the ADR accumulation, but not NK-2280 accumulation.

Materials and Methods

Cell lines and cultures. EAT cells, its ADR-resistant strain, NIH3T3 cells (a mouse fibroblast cell line which had been transfected with several oncogenes via the by pSV2neo vector) (2), were used in this study. An ADR-resistant EAT strain, derived from the wild EAT strain, was grown in medium containing 0.2, 0.5, 1, 5 and 10 μg/ml ADR and established in our laboratory. The wild EAT strain and NIH3T3 cell lines were subcultured in Dulbecco's modified Eagle medium (DME medium) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 100 units/ml of penicillin (Meiji Seika Co., Ltd., Tokyo, Japan), 100 μg/ml of streptomycin (Meiji Seika), and 10% bovine calf serum (Hyclone Laboratories, Inc., UT, USA) at 37°C using a CO_2 incubator supplied with 5% CO_2 + 95% air. The ADR-resistant EAT strain was similarly subcultured in a medium which also contained 0.2, 0.5, 1,5 and 10 μg/ml ADR, and was returned to the ADR-free medium for 1 week before the experiment.

Drugs. ADR (Kyowa Hakko Kogyo, Co., Ltd., Tokyo, Japan) was dissolved in distilled water to 1 mg/ml. NK-2280 (Nippon Kankoh Shikiso Kenkyusho Co., Ltd., Okayama, Japan) was dissolved in 99.5% ethanol. They were frozen, and thawed immediately before use, then made to the correct concentration with DME medium. They were handled under yellow light. Amiloride (Sigma Chemical Co., Ltd., St. Louis, MO, USA) and DIDS (Sigma Chemical) were dissolved in distilled water, and made to the correct concentration with DME medium prior to use in the experiment.

Measurement of intracellular accumulation of ADR. ADR emits fluorescence with peaks at 556 and 582 nm following excitation at 488 nm. The intensity of intracellular fluorescence per cell was measured by flow cytometry (FCM). Cells were seeded in a 15 ml test tube (Becton Dickinson and Company, New Jersey, USA), which was placed in water at 37°C. The cells were then exposed to ADR, rinsed with cold phosphate-buffered saline three times, (NIH3T3 dispersed with trypsin centrifuged, cooled at 0° C, and filtered through a 40 μm nylon mesh. Intracellular fluorescence was measured by FCM using an EPICS PROFILE II (Coulter Corporation Hialeah, FL., USA). Excitation was done at 488 nm, and the intensity of fluorescence at 575-625 nm was measured in 20,000 cells. The mean intensity of fluorescence was calculated from obtained histograms. From this value, the mean intensity of fluorescence in ADR-untreated control cells was subtracted, and the mean intensity of fluorescence from intracelular ADR was estimated (14, 15).

Measurement of cell membrane potential by NK-2280. NK-2280 emits fluorescence with a peak at 495 nm following excitation at 488 nm. The intensity of the intracellular accumulation of NK-2280 fluorescence at 500 nm was measured by methods similar to the those used measurement of ADR accumulation after excitation at 488 nm (16).

Results

Firstly, the intracellular accumulation of ADR and NK-2280

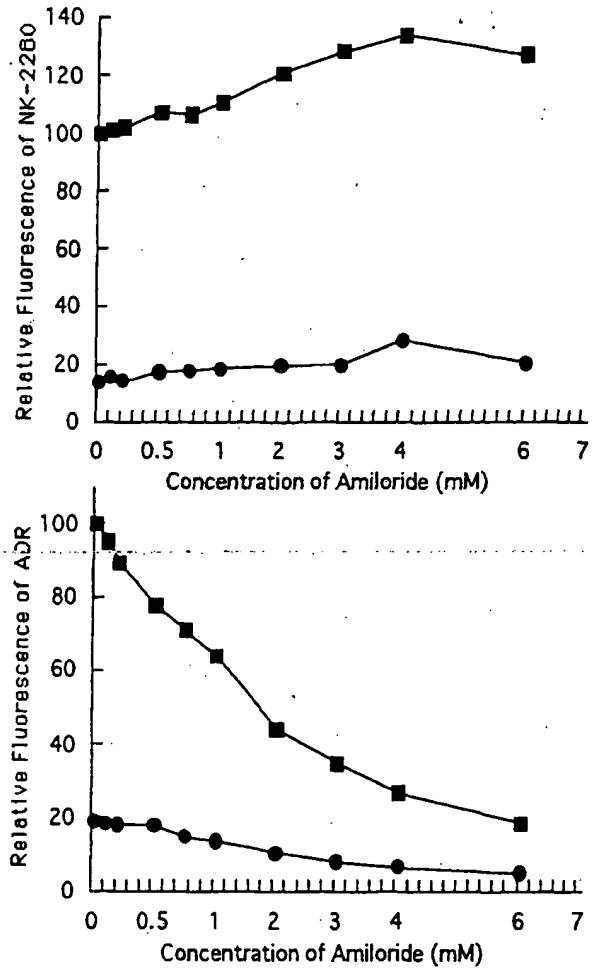

Figure 3. *Influence of amiloride on the intracellular accumulation of NK-2280 (upper) and ADR (lower) in the wild and the 1 μg. ADR-resistant EAT strains.*
The wild EAT cells (■) and the 1 μg ADR-resistant EAT cells (●) were treated with 10 μg/ml ADR for 60 min, or 0.5 μM NK-2280 for 20 minutes at 37° C while the concentration of amiloride was 0.1, 0.2, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, 6.0 mM.

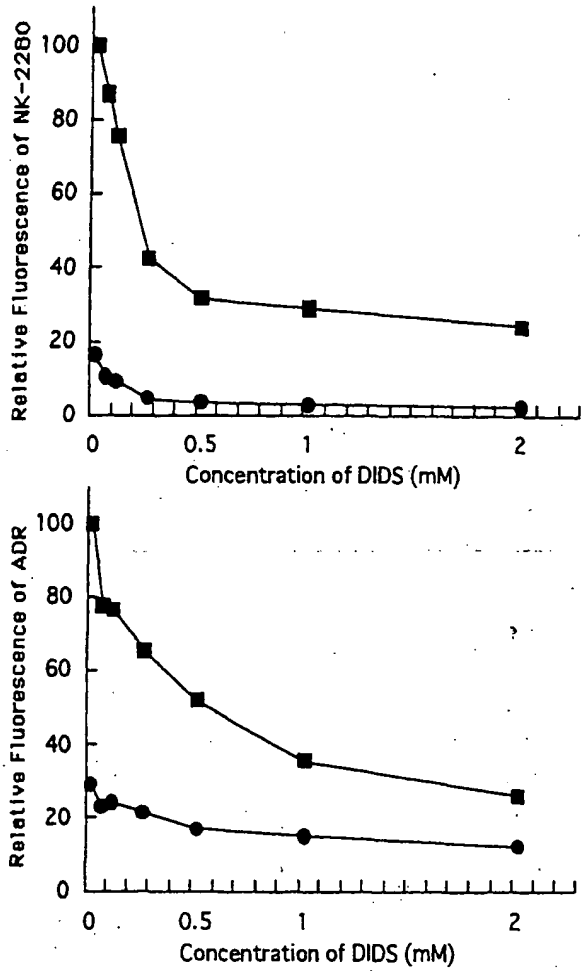

Figure 4. *Influence of DIDS on the intracellular accumulation of NK-2280 (upper) and ADR (lower) in the wild and the 1 μg ADR-resistant EAT strains.*
The wild EAT cells (■) and the 1 μg ADR-resistant EAT cells (●) were treated with 10 μg/ml ADR for 60 min, or 0.5 μM NK-2280 for 20 min at 37° C while the concentration of DIDS was 0.05, 0.1, 0.25, 0.5, 1.0, 2.0 mM.

in wild and ADR-resistant EAT strains and NIH3T3 cell lines were observed. In the NIH3T3 cell lines transfected with several oncogenes accompanied by pSV2neo vector, a good correlation was observed between increases in ADR accumulation and NK-2280 accumulation (Figure 1) ($r=0.838$, $p< 0.0005$). In the EAT strains, a good correlation was also found between increases in ADR accumulation and NK-2280 accumulation (Figure 2) ($r=0.993$, $p<0.0001$).

Next, the influence of amiloride and DIDS on the intracellular accumulation of ADR or NK-2280 was also examined in the wild EAT strain and the 1 μg ADR-resistant strain. ADR accumulation decreased, but NK-2280 accumulation increased with increasing concentratios of amiloride in both EAT strains (Figure 3). ADR and NK-2280 accumulation in both EAT strains decreased with increasing concentratios of DIDS (Figure 4).

Discussion

In the present study, we first investigated the intracellular accumulation of ADR and NK-2280, an indicator of cell membrane potential, in several cell lines. In the NIH3T3 cell lines transfected with several oncogenes accompanied by pSV2neo vector, there was a good correlation between increases in ADR and NK-2280 and intracellular accumulation ($r=0.838$, $p< 0.0005$) (Figure 1). Further more,in the

EAT strains there was also a good correlation between increases in ADR accumulation and NK-2280 accumulation ($r=0.993$, $p< 0.0001$) (Figure 2). Hasmann has shown that the increase in drug accumulation of a number of positively-charged fluorescent dyes, such as Rhodamin 123, Rhodamin 6G, Saffranin O and ADR, correlated with increased the cell membrane potential by using positively-charged cell membrane potential fluorescent probes; 3,3'-dihexyl-oxacarbocyanine (DiOC6(3))(8). This may show that ADR accumulation is influenced by cell membrane potential, or that, the mechanisms for intracellular accumulation of NK-2280 may be similar to those of ADR.

We also observed the influence of the Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger on the intracellular accumulation of ADR and NK2280. The ADR accumulation decreased, but NK-2280 accumulation increased with increasing concentratios of amiloride, an ihibitor of the Na^+/H^+ exchanger (11, 12) (Figure 3). Further more, both ADR accumulation and NK-2280 accumulation decreased with increasing concentrations of DIDS, an inhibitor of the Cl^-/HCO_3^- exchanger (13) (Figure 4). The increased accumulation of NK-2280 caused by amiloride was considered to be due to the increased cell mem brane potential, caused by the inhibition of H^+ ion efflux or Na^+ ion influx. The decreased accumulation of NK-2280 caused by DIDS may be also due to the decreased cell membrane potential due to the inhibition of Cl^- ion efflux by DIDS. However, the decreased rate caused by DIDS was greater than the decreased rate caused by amiloride. Therefore, it is suggested the decreased accumulation of NK-2280 by DIDS may be influenced by other factors than membrane potential. The decreased accumulation of ADR by amiloride or DIDS, and that of NK-2280 by DIDS may be due to inhibition of either the Na^+/H^+ exchanger or the Cl^-/HCO_3^- exchanger. These results suggest that the ADR accumulation may be related to both the Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger, but that NK-2280 accumulation may be related to the Cl^-/HCO_3^- exchange alone. Therefore, we suggest that the Cl^-/HCO_3^- exchanger is of low selectivity for drug accumulation. It is interesting that the influx ability of the Cl^-/HCO_3^- exchanger as well as the efflux ability caused by P-glycoprotein is of low selectivity, since it has been reported that P-glycoprotein is related to a chloride channel.

In the present study, we first examined the intracellular accumualtion of ADR and NK-2280, and found a good correlation between them. This suggests that ADR accumulation may be influenced by cell membrane potential or that the mechanisms of NK-2280 accumulation may be similar to those of ADR accumulation. Next, we observed the influence of Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger on the intracellular accumulation of ADR and NK-2280, and found that ADR accumulation was influenced by both exchangers, but that NK-2280 accumulation was influenced by the Cl^-/HCO_3^- exchanger alone. This suggests that the Cl^-/HCO_3^- exchanger may be of low selectivity on the drug accumulation.

Acknowledgements

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